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Applicant: DIECKMANN et al. Conf. No.: 8230

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For: ANTISENSE COMPOUNDS, METHODS AND COMPOSITIONS FOR TREATING MMP-12 RELATED INFLAMMATORY DISORDERS

L E T T E R

Assistant Commissioner for Patents
P.O. Box 1450
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Date: February 6, 2004

Sir:

Under the provisions of 35 U.S.C. § 119 and 37 C.F.R. § 1.55(a), the applicant(s) hereby claim(s) the right of priority based on the following application(s):

Country	Application No.	Filed
SWEDEN	0202253-1	July 18, 2002

A certified copy of the above-noted application(s) is(are) attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fee required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

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Attachment

(Rev. 04/19/2000)

PRV

PATENT- OCH REGISTRERINGSVERKET
Patentavdelningen

Intyg
Certificate

Härmed intygas att bifogade kopior överensstämmer med de handlingar som ursprungligen ingivits till Patent- och registreringsverket i nedannämnda ansökan.

This is to certify that the annexed is a true copy of the documents as originally filed with the Patent- and Registration Office in connection with the following patent application.

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Novel sequence information and methods for its use in diagnosis and therapy XIII

Field of the invention

The present invention relates to changes in gene expression associated with inflammatory diseases, and in particular inflammatory bowel diseases, gene sequences identified to have such association, and to methods of using such genes or gene products as tools in research, diagnosis or prognosis, or as targets for therapeutic intervention.

Specifically, this invention relates to a novel human gene sequence, which has been identified in colonic mucosa from eight (8) individual patients positively diagnosed with Ulcerative colitis (UC), a subgroup of inflammatory bowel disease. This sequence can *inter alia*: 1) serve as a marker for UC; 2) serve as a marker for inflammatory diseases; and 3) provide means of identifying agents that modulate their expression in colonic mucosa cells and other tissues such that beneficial effects with respect to inflammation can be achieved in a patient.

Background of the invention

Inflammatory bowel disease (IBD) comprises several conditions involving chronic inflammation in the gastrointestinal tract. The aetiology of IBD is however poorly understood, and several lines of evidence indicate disordered immune regulation, generation of soluble mediators of inflammation and stimulation by intestinal luminal antigens (e.g., bacteria) as being critical factors. Two of the most debilitating forms of IBD are UC and Crohn´s disease (CD). Both UC and CD are recognised as a major health problem of global proportions and there is currently no cure for either condition.

These diseases affect young people, with a typical debut at the age of 20-30 years, and disease management is a long-term commitment for both patient and physician. Whereas the inflammation of UC is restricted to the mucosa and submucosa of colon and rectum, CD shows a wide spread inflammation of the gastro-intestinal tract with granuloma formation.

Current drug treatment regimes focus largely on symptomatic control, but patients often experience periods of relapsed symptoms in the form of nausea, vomiting, bloody diarrhoea, severe abdominal pain and weight loss.

Patients with long-standing UC are at considerable risk (up to 20 fold over non-UC cases) of developing colorectal cancer and some 50-60 % of IBD patients will require surgical intervention 7-10 years after diagnosis. Resection of the affected area and administration of cytostatic drugs constitute the first line of treatment, however only 30 % of patients respond positively to drug therapy. This equates to a large proportion of patients needing either further surgical treatment or refined medical treatment programs.

To clinically distinguish UC from colonic CD at an early stage would provide enormous benefits for both the physician and the patient. It would permit the design of accurate treatment regimes, prevent unnecessary medications and reduce treatment costs. Even though the overall clinical picture in IBD patients may show some clinically important differences between the major patient groups of UC and CD, there are substantial similarities, thus making it difficult for health care personnel to establish the correct diagnosis. A diagnostic tool that allows discrimination of high cancer risk UC patients from other IBD patients would have significant impacts on the general welfare of the patient, the form of therapeutic regimes administered and if required, the type of surgical intervention deemed most appropriate.

One way to accurately assess the early manifestation of UC is to identify markers, which are uniquely associated with disease progression. Likewise, the development of therapeutics to prevent or repair colon damage relies on the identification of genes responsible for colon cell growth and function.

It remains to identify relevant and reliable marker for inflammatory diseases, especially IBDs, and to develop diagnostic, prognostic, and therapeutic tools for such diseases.

Summary of the invention

The present invention is based on the discovery of a novel sequence, differentially expressed in active UC when compared to CD or non-diseased colonic mucosa. The

invention encompasses the isolation of nucleic acid molecules or cDNA molecules as defined in claim 1, and in particular SEQ ID NO. 1, whose expression is altered in humans as a consequence of manifested inflammatory disease, in particular inflammatory bowel diseases.

The invention further includes the foresaid nucleic acid molecules operably linked to one or more expression control elements. Including but not limited to prokaryotic and eukaryotic vectors harbouring the isolated nucleic acid molecules.

The invention also includes host cells transformed with these sequences, as well as transgenic animals having these sequences functionally inserted, over-expressed or suppressed, in at least one of their cells. Such animals are useful as research tools for investigations regarding the aetiology of IBDs, the progression, diagnosis and treatment of the same.

The invention in particular provides agents and methods for the modulation, preferably the inhibition, of the expression of the genes identified by the sequence SEQ. ID. NO. 1.

The invention is further defined in the attached claims, incorporated herein by reference.

Brief Description of the Drawings

The invention will be described in closer detail in the following description, examples and figures, in which

Fig. 1 (drawings not submitted at date of priority)

Description of the invention

Before the present method is disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope

of the present invention will be limited only by the appended claims and equivalents thereof.

In the description, examples and claims, the following terms will be used (defined in alphabetical order):

"Antisense" as in "antisense molecules" and "antisense sequences" referes to single stranded RNA or DNA molecules complementary to a portion of the mRNA of a target gene. The antisense molecule will base-pair with the mRNA, thus preventing translation of the mRNA into protein. Consequently, the term "antisense therapy" refers to methods using such antisense compounds which specifically hybridise to a target nucleic acid and modulate its function or translation, for example by suppressing or reducing the expression of gene products coded by said sequence.

"Complementary" in the context of this description refers to the capacity for precise pairing between two nucleotides.

"Hybridisation" means bonding between complementary nucleoside or nucleotide bases. "Hybridisation under stringent conditions" refers to the criteria regarding temperature and buffers well know in the field.

"Functionally homologous" means sequences sharing perhaps a lower structural homology with the disclosed sequences, but exhibiting homologous function in vivo, in either the healthy or the diseased organism, e.g. coding the same or highly similar proteins.

"Functionally inserted" or "operationally inserted" denotes that a sequence has been inserted in a host genome in such orientation, location and with such promoters, where applicable, that the correct expression of said sequence occurs. "Modulation" as used in this context means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

"PNA" or peptide nucleic acids are synthetic chimeras of nucleobases linked to a peptide backbone.

SEQ. ID. NO. 1: This patent refers to a gene called metalloproteinase 12 (MMP12). Matrix metalloproteinases (MMPs) are a family of zinc-dependent neutral endopeptidases collectively capable of degrading essentially all matrix components. They seem to play an essential role in cancer progression and inflammation. MMP-12 is also called macrophage metalloelastase and it has now surprisingly been shown that this enzyme is upregulated in IBD.

The identified sequence (SEQ. ID. NO. 1) seems to match Acc# XM_058069.

Specific Embodiments

Diagnostic applications

The sequences disclosed herein, and information derivable thereof, such as gene fragments and full length sequences identifiable using the described sequence (SEQ. ID. NO. 1), enables new diagnostic approaches. By determining the presence or absence of such sequence, or the expression or translation of the same, indications to the presence or absence of inflammatory states can be obtained. The nucleic acid molecules, or proteins or antibodies derived thereof, according to the present invention may be labelled or conjugated to a variety of labels or other molecules, normally employed in diagnostic applications, such as fluorescent markers, enzymatic markers, toxic molecules, molecules which are non-toxic but become toxic upon exposure to a second compound, and radionuclides.

Diagnostic methods according to the invention also involve the detection of low level expression of said sequence (SEQ. ID. NO. 1) or gene fragments or full length sequences identifiable through the sequence disclosed herein. Patient samples, such as biopsy samples, samples of body fluids such as blood, serum, lymphatic fluid, urine or faeces may be utilized, and the presence or absence of gene expression determined according to methods well known in the art. In many cases, the availability of sample may be limited,

and methods of *in vitro* amplification of the cDNA may have to be resorted to. An example of such methods is given in the Examples section of the present description.

Prognostic applications

Progostic applications of the inventive sequences involves the detection of low level expression of said sequence (SEQ. ID. NO. 1) or gene fragments or full length sequences identifiable through the sequences disclosed herein. Patient samples, such as biopsy samples, samples of body fluids such as blood, serum, lymphatic fluid, urine or faeces may be utilized, and the presence or absence of gene expression determined according to methods well known in the art. In many cases, the availability of sample may be limited, and methods of *in vitro* amplification of the cDNA may have to be resorted to. An example of such methods is given in the Examples section of the present description.

Therapeutic applications

The present invention provides different therapeutic methods, based on the regulation of the genes, identified by the sequence of the present invention (SEQ. ID. NO. 1), including methods for the down-regulation or suppression of the expression of said genes.

Antisense technology is emerging as an effective means for reducing or suppressing the translation of specific genes and the expression of specific gene products, and may therefore be useful for the treatment of inflammatory diseases, based on the specific sequences disclosed in the present description. The present invention thus provides methods and compositions for use in antisense therapy.

In particular, the present invention provides a compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule identified by comprising a sequence according to SEQ. ID. NO. 1, or parts thereof, wherein said compound hybridises with and modulates, preferably inhibits, the expression of said nucleic acid molecule.

Said compound should be able to exhibit specific and stable hybridisation, and preferably said compound comprises 8 to 24 nucleobases in length. Most preferably, said compound has a sequence comprising at least an 8-nucleobase portion of SEQ. ID. NO. 1.

According to a preferred embodiment, said compound is an antisense oligonucleotide. According to a further embodiment, said antisense oligonucleotide comprises at least one modified nucleobase, which may be chemically modified by substitution in a non-bridging oxygen atom of the antisense nucleic acid backbone with a moiety selected from the group consisting of methane phosphate, methyl phosphate and phosphorothioate. According to one embodiment, said substitution is at one or more nucleotides independently selected from the final three nucleotides at the 3' terminus and 5' terminus of said oligonucleotide. It is also conceived, that the substitution occurs at any position along the entire length of said oligonucleotide. Preferably, said oligonucleotide comprises at least one modified sugar moiety nucleobase, and the modified sugar moiety may be a 2'-O-methoxyethyl sugar moiety.

Said antisense agent may also be an antisense agent composed of DNA or RNA or an analogue or mimic of DNA or RNA including but not restricted to the following: methylphosphonate, N3'->P5'-phosphoramidate, morpholino, peptide nucleic acid (PNA), locked nucleic acid (LNA), arabinosyl nucleic acid (ANA), fluoro-arabinosyl nucleic acid (FANA) methoxy-ethyl nucleic acid (MOE). Preferably said antisense agent is a homo or heteropolymer containing combinations of the above DNA or RNA or analogues or mimics of DNA or RNA.

The present invention also makes available a pharmaceutical composition, wherein said composition comprises a compound or antisense agent as describe above, and a pharmaceutically acceptable carrier or diluent. Said pharmaceutical composition preferably further comprises a colloidal dispersion system.

The present invention also makes available a method of inhibiting the expression of a gene identified by comprising a sequence according to SEQ. ID. NO. 1 in human cells and tissues, comprising said human cells and tissues *in vivo* or *in vitro*, wherein that a compound or antisense agent as described above is used.

The present invention also provides a variety of pharmaceutical compositions, comprising eukaryotic cells transformed with any of the disclosed sequences, SEQ. ID. NO. 1, along with a pharmaceutically or physiologically acceptable carrier, excipients or diluents.

Generally, such carriers should be non-toxic to the recipient at the dosages and concentrations used. Ordinarily, the preparation of such compositions involves combining the therapeutic agent with one or more of the following: buffers, antioxidants, low molecular weight polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with non-specific serum albumin are examples of suitable diluents. Such compositions may find use in gene therapy applications. Such compositions according to the present invention may be administered in a number of ways depending on whether local or systemic treatment is desired. Administration according to the present invention is preferably topical, and local, as in rectal or oral delivery to the mucous membranes of the gastrointestinal tract. Formulations for gastrointestinal administration include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners etc may be used as desired. Solutions, emulsions and liposome-containing formulations are included. A preferred form of administration is a liquid, preferably water based formulation, administered rectally in the form of an enema.

Gene therapy according to the present invention is not limited to the treatment of a manifest disease, in particular an inflammatory bowel disease, but also encompasses the prevention of the onset or recurrence of such diseases.

Further, antisense compounds are commonly used in research and diagnostics. Antisense oligonucleotides, which are able to inhibit gene expression with great accuracy, may be used to elucidate the function of particular genes and thereby offer novel therapeutic strategies for the treatment of inflammatory disorders. The present invention encompasses such applications of antisense compounds, based on the information contained in the sequences disclosed herein, or directed to genes identifiable therewith.

Transgenic cells and animals

The present invention also provides transgenic cells as such, as well as transgenic animals. Transgenic animals include animals comprising viable transgenic cells, or

transgenic organs, as well as entire animals incorporating any one of the inventive sequences (SEQ. ID. NO. 1) or functional parts thereof, in their genome. The inventive sequences may be over-expressed, suppressed or unregulated in said transgenic cells, organs or animals.

One embodiment of the present invention is thus such transgenic cells, organs or animals, and their use as models for investigating the nature and/or aetiology of inflammatory diseases, in particular IBDs, as models for evaluating the efficacy of pharmaceuticals against such diseases, as well as investigating the effect of known and suspected causative agents behind such diseases.

Examples

Identification of the appropriate biopsy material

The biopsies were taken from patients who were selected on the basis of clinical and pathological anatomical evidence of having UC, with or without the complication of colorectal dysplasia or cancer. A total of three (3) biopsies per anatomical site are collected from the colon of a single individual patient. That is 3 biopsies from the active inflamed site (active target tissue) and 3 biopsies from non-inflamed area (normal control tissue). This was done for eight (8) different patients.

Isolation of differentially expressed total RNA

The biopsies of each anatomical site of one patient was pooled and total RNA was isolated using Quiagen Rneasy Kit and a Pellet Pestel Motor Homogenizer according to the manufacturers protocol. In this way 16 samples of total RNA were isolated, two samples per patient: inflamed (target) and non-inflamed (control).

Performing cDNA synthesis of the RNA

Two microgram of each RNA sample (16) was used for a first strand cDNA synthesis using 10pM of the Oligo-dT-primer dT-joint (5'-TAG TCT ATG ATC GTC GAC GGC TGA TGA AGC GGC CGC TGG AGT TTT TTT TTT TTT TTT TTV-3' (SEQ. ID. NO. 101)) introducing to every synthesised cDNA molecule three restriction enzyme cutting

sites: SalI, NotI and BpmI. The buffer, the desoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP) and the enzyme reverse transcriptase (Superscript II) were taken from Gibco BRL and the reactions were done as described in the protocol supplied with the material. The reaction mixture for first strand synthesis excluding the enzyme was preincubated for 5 min at 65°C in a PCR machine (PCR sprint from Hybaid), chilled on ice, preheated to 42°C, the enzyme Superscript II was added and incubated for 1h at 42°C in a PCR machine (PCR sprint from Hybaid).

For the second strand synthesis 41 µl second strand buffer mix were added to the reactions according to the provided protocol (Gibco BRL) and 4µl *E.coli* Polymerase I (New England Biolabs), 1.5 µl *E.coli* DNA ligase (New England Biolabs) and 0.7 µl Rnase H (Gibco BRL) in a total volume of 160 µl. These reactions were incubated for 2.5h at 16°C in the PCR machine PCRsprint and then purified using the Quiagen PCR Purification Kit according to the protocol provided. Every sample (16 in total) was eluted with 32 µl of elution buffer and 26 µl of each sample was used for the following steps.

Amplification of the 3'-termini of the cDNAs

The amount of material isolated and processed out of biopsies is very restricted. Consequently, the present inventors used the new method described in co-pending application SE 0200035-4, incorporated herein by reference, for *in vitro* amplification of the 3'-end of cDNA.

For *in vitro* amplification of the 3'-end of cDNA 26 µl cDNA of every sample (16 in total) was digested with 10U of the restriction enzyme DpnII in a volume of 30 µl for 3h at 37°C. Then the cut cDNAs were purified once more using Quiagen PCR purification Kit and the cDNAs were eluted in 47 µl elution buffer. The following circular ligation step was performed in a volume of 50 µl including 44 µl of the DpnII cut cDNA and 2000U T4 DNA ligase (New England Biolabs). These reaction mixtures were incubated at 22°C for 1h, heat inactivated by 65°C for 10 min and 25 µl of each reaction mixture was used for the amplification step. A mixture for 5 reactions per sample was put together (5x 50 µl= 250 µl in total) containing 25 µl cDNA (DpnII cut and circular

ligated), 25 µl 10x Advantage 2 PCR buffer (Clontech), 5 µl joint-Not primer (10 pmol/µl; 5'-TGA TGA AGC GGC CGC TGG-3' (SEQ. ID. NO. 102)), 5 µl joint-Sal primer (10pmol/µl; 5'- TTC ATC AGC CGT CGA CGA TC –3' (SEQ. ID. NO. 103), 5 µl 10mM dNTP mix and 5 µl 50x Advantage 2 Taq-Polymerase (Clontech). For each sample (16 in total) the mix was distributed into 5 PCR reaction tubes and PCR performed under the following conditions: 1 min 94°C then 16x (20 sec 94°C, 20 sec 55°C, 1 min 72°C).

Four of the reactions per sample were removed and placed on ice and the optimal cycle number was determined with one of the reactions per sample. The optimal cycle number was determined to 18 cycles for all 16 samples, thus for the remaining four reactions per sample two additional cycles [2x (20 sec 94°C, 20 sec 55°C, 1 min 72°C)] were performed. The 4 PCR reactions per sample were subsequently purified using the Quiagen PCR purification Kit. For the purification, the four reactions per sample were pooled (total of 200 µl) and then eluted with 34 µl elution buffer. The purified reactions were the working material for the identification of the differentially expressed genes.

Isolation of the differentially expressed cDNA (subtraction protocol)

To isolate differentially expressed genes via a PCR based subtraction method different primer adaptors have to be ligated to a part of the cDNA. Thus, cDNA has to be prepared. A second goal for the following processing of the cDNA is to remove the Oligo-dT tail for a more specific subtraction.

For the preparation of the adaptor ligation 26 µl of the cDNAs was Sal I digested in a volume of 30 µl with 10U SalI for 2h at 37°C. The cDNAs were then purified using the Quiagen PCR purification Kit and eluted with 35 µl elution buffer. Then a partial fill in reaction was performed for the ligation of the 5' adaptors by taking 33 µl of each cDNA sample (SalI cut) and incubating it for 1h at room temperature with 20U of the Klenow fragment of DNA polymerase in the presence of dTTP (1µl of 10 mM in 40 µl volume) to destroy the SalI ends, which will be restored in the part of the cDNA which the 5' adaptors will be ligated to. The samples were then purified again using the Quiagen PCR

purification kit, eluted with 36 µl of elution buffer and 33 µl was used for a BpmI digest to remove the Oligo-dT tail and to prepare the 3' ends for the ligation of the 3' adaptors. The cDNAs were incubated with 6U BpmI for 2h at 37°C. After the next purification step with Quiagen PCR purification kit, the concentration of the cDNAs were estimated on a 1% agarose gel so that the concentration of the cDNAs of one patient, i.e. both target and control, are approximately the same.

Adaptor ligation to a part of the cDNA samples ("tester" cDNA generation)

To have the possibility to enrich the genes that are differentially expressed in all eight patients, the present inventors developed a PCR amplification system ("pyramide; described below). To perform this part, two different 5' and 3' adaptors (instead of one each) were ligated to the ends, which differ from usual PCR based subtraction protocols.

Table 1 shows the volume in µl of each cDNA sample used for the adaptor ligation and with which adaptors each patient cDNA was ligated. One ligation reaction contained the cDNA of one sample (16 in total) and from each sample two reactions were performed (with two different adaptors), making it a total of 32 reactions. In one reaction 40 pM adaptor and 2000U of T4 Ligase (New England Biolabs) was used and the reactions were incubated for 4h at 22°C.

Table 1.

Patient	Sample type	Applied cDNA	Adaptor
Patient A	c	4	1+2
	t	2	1+2
Patient B	c	2	1+2
	t	2	1+2
Patient C	c	2	1+2
	t	2	1+2
Patient D	c	2	1+2
	t	4	1+2
Patient E	c	2	3+4
	t	2	3+4
Patient F	c	2	3+4
	t	2	3+4
Patient G	c	2	3+4
	t	2	3+4

Patient H	c	2	3+4
	t	2	3+4

For the adaptor ligation the adaptors had to be made out of two oligonucleotides, which were annealed to form double strand molecules. The oligonucleotide sequences were:

Ada-1L: 5'- AAC CAC CTG CGA GTA CAA TCT GGG CGG CCG CAG TCT T-3' (SEQ. ID. NO. 103)

Ada-2L: 5'- GCA CTC GTA TGG TTG TAG TTG CGG CGG CCG CAG TCT T-3' (SEQ. ID. NO. 104)

Ada-3L: 5'- TCG TTA GCG TGC TGT CAT TCC TGG GGG CCG CAC AG-3' (SEQ. ID. NO. 105)

Ada-4L: 5'- GGA ACA GTA GCA GGG TTT GTC TCG GGG CCG CAC AG-3' (SEQ. ID. NO. 106)

Ada-1/2S: 5'- GAC TGC GGC CG-3' (SEQ. ID. NO. 107)

Ada-3/4S: 5'- CGA CTG TGC GGC CC-3' (SEQ. ID. NO. 108)

The oligonucleotides Ada-1L and Ada-2L were annealed to the oligonucleotide Ada-1/2S to get adaptors Ada1 and Ada2. To form adaptors Ada3 and Ada4 the oligonucleotides Ada-3L and Ada-4L were annealed to the oligonucleotide Ada-3/4S. This was done in 40 ml reactions where 18 ml of 200 pmol/ml stock solution of both oligonucleotides in 1x SalI buffer were placed into a sealed tube and then submerged in 1 ltr of boiling water and subsequently put in room temperature for 5-6h for cooling down. After this procedure, 5 ml of each annealed adaptor was sampled, and 45 ml water added to provide a stock solution of 20 pmol/µl, which was then used in the adaptor ligation reaction.

After the ligation reaction 16 µl 10 mM Tris pH 8.0 was added to the cDNA samples ("tester cDNA", 32 in total).

Driver cDNA generation

The rest of the purified BpmI digested cDNA samples was lyophilized by incubation with an open lid in a heating oven at 50°C. The pellets were then dissolved in water in order to become 2x concentrated with respect to the tester cDNA (see table 2).

Table 2: Amount of applied water (µl)

Patient	Sample type	Applied water (µl)
Patient A	c	7.5
	t	13
Patient B	c	13
	t	7.5
Patient C	c	13
	t	13
Patient D	c	13
	t	13
Patient E	c	13
	t	13
Patient F	c	13
	t	13
Patient G	c	7.5
	t	13
Patient H	c	13
	t	13

Subtractive Hybridisation

In order to subtract the non-differentially expressed genes from the differentially expressed genes, the target cDNA (tester) was subtracted against an excess of control cDNA (driver). The subtraction reaction is a normal hybridisation step of two denatured cDNAs, which can only anneal at high temperature because they share at least 70% identity. The present inventors performed two subtractions per patient, in total 16 subtractions. For each subtraction two hybridisation steps were performed. In the first hybridisation the driver cDNA is incubated with only one type of tester cDNA, which means two reactions, A and B, per sample and subtraction (see tab 3). In table 3 the composition of the reactions for the first hybridisation step is shown. Altogether this were

32 reactions; Ada A stands for Ada 1 or 3 and Ada B stands for Ada 2 or 4 (see also tab 2). The reactions were incubated at 98°C for 1.5 min in PCR tubes and then at 68°C for 8-9h.

Table 3. Composition of the reactions in the first hybridisation step

	Reaction A	**Reaction B**
Driver	1.5 µl	1.5 µl
Tester (+ Ada A)	1.5 µl	/
Tester (+ Ada B)	/	1.5 µl
4 x Hyb buffer	1 µl	1 µl
Mineral oil	10 µl	10 µl

In the second hybridisation step the two reactions, A+B, were combined and additional driver cDNA was added. To perform this step, fresh PCR tubes were used and 0,5 µl driver (target or control as before depending on the subtraction), 0,5 µl 4xHyb buffer, 1 µl H_2O and 6 µl mineral oil was added to the tubes and subsequently incubated at 98°C for 1,5 min. The reactions from the first hybridisation were then added to the new PCR tubes, so that reactions A+B were combined with new excess of driver (16 reactions in total), and the combined samples incubated over night at 68°C.

In order to remove the annealed fragments with lower identity, 200 ml of dilution buffer (20 mM Hepes pH 8.0, 50 mM NaCl, 0.2 mM EDTA) was added and incubated for an additional 10 min at 68°C.

PCR amplification of enriched genes

After this subtraction procedure the non-subtracted specific gene fragments were amplified using the ligated adaptors (see tab.1) to prime the oligonucleotides. The

subtracted cDNA of patients A to D was amplified with the oligo primers ad1 and ad2 and the cDNA of patients E-H with the primers ad3 and ad4 (Note: for each patient two reactions were done for up and down regulation giving a total of 32 reactions).

The sequences of the used oligonucleotide primers are:

Ad1: 5'-AAC CAC CTG CGA GTA CAA TCT GG-3' (SEQ. ID. NO. 109)

Ad2: 5'-TCG TTA GCG TGC TGT CAT TCC TG-3' (SEQ. ID. NO. 110)

Ad3: 5'-GCA CTC GTA TGG TTG TAG TTG CG-3' (SEQ. ID. NO. 111)

Ad4: 5'-GGA ACA GTA GCA GGG TTT GTC TC-3' (SEQ. ID. NO. 112)

For the PCR reaction 5 ml of the hybridisation sample was used, 5 µl of the 10x Advantage 2 PCR buffer of Clontech, 1 µl of primer ada 2 or 4, 1 µl of primer ada 1 or 3, 1 µl of 1 mM dNTP mix and 1 µl 50xAdvantage 2 Taq polymerase of Clontech in a total volume of 50 µl. The cycling parameters were 75°C for 3 min, 94°C for 1 min and then 28 times 94°C for 20 sec, 68°C for 20 sec and 72°C for 1 min.

Two reactions per sample were combined and 5 µl run on an agarose gel, the remaining 95 µl was purified using Quiagen PCR purification kit and eluted with 30µl elution buffer. Of the amplified and purified cDNAs were 20 µl used as the material for the pyramid enrichment.

<u>Further enrichment of the differentially expressed genes (pyramid protocol)</u>

After the first amplification step (described in "PCR amplification of enriched genes") one adaptor was cut off from one end of the cDNA fragments of each patient and then cDNA from two patient were combined in a way that after the hybridisation step and refill step only the these cDNAs that were enriched in both patients could be amplified. For example, patient A has ada2 as 5'-adaptor and ada1 as 3'-adaptor, patient B has ada4 as 5'-adaptor and ada3 as 3'-adaptor. The cDNA of patient A was cut with SalI which will only cut the 5'-adaptor, and the cDNA of patient B was cut with NotI, which

likewise, will only cut the 3'-adaptor. When they are mixed and hybridised, only the cDNAs that are enriched in both patients could find each other and be amplified after a fill-in reaction. The cDNAs that are only enriched in one patient find their complementary strand, but they are missing the second adaptor for amplification. The new "hybrids" can then be amplified with the oligo primers ad4 (5'-end primer) and ad1 (3'-primer). The individual steps of the pyramid are shown in tab.4. The pyramid was performed twice for up- and down subtraction.

Table 4. Scheme of the pyramid protocol

Reaction	Patient A	Patient B	Patient C	Patient D	Patient E	Patient F	Patient G	Patient H
Amplification 1	2 + 1	4 + 3	1 + 2	4 + 3	2 + 1	4 + 3	2 + 1	4 + 3
Digest 1	SalI	NotI	NotI	SalI	NotI	SalI	SalI	NotI
Hybridisation 1	Patient samples A + B		Patient samples C + D		Patient samples E + F		Patient samples G + H	
Amplification 2	4 + 1		2 + 3		2 + 3		4 + 1	
Digest 2	SalI		NotI		SalI		NotI	
Hybridisation 2	Patient samples A + B + C + D				Patient samples E + F + G + H			
Amplification 3	2 + 1				4 + 3			
Digest 3	SalI				NotI			
Hybridisation 3	Patient samples A + B + C + D + E + F + G + H							
Amplification 4	4 + 1							
Digest 4	SalI + NotI							

Digest protocol:

For each digest 20 ml of 30 ml purified cDNA was used in a volume of 30 ml. The cDNA was digested with 10U of SalI or NotI for 3h at 37°C and subsequently heat inactivated for 20 min at 65°C.

Hybridisation protocol:

Three ml of sample A was combined with 3 ml of sample B in 1x hybridisation buffer in a volume of 8 ml, covered with 15 ml mineral oil. The reaction was performed in a PCR machine for 13h at 68°C. The reaction was then diluted with 500 ml dilution buffer and 5 ml was used for the next PCR amplification step.

Amplification protocol:

For the PCR reaction 5 ml of the hybridisation sample was used, 5 µl of the 10x Advantage 2 PCR buffer of Clontech, 1 µl of primer ada 2 or 4, 1 µl of primer ada 1 or 3, 1 µl of 1 mM dNTP mix and 1 µl 50xAdvantage 2 Taq polymerase of Clontech in a total volume of 50 µl. The cycling parameters were 75°C for 3 min, 94°C for 1 min and then 13 times 94°C for 20 sec, 68°C for 20 sec and 72°C for 1 min. The PCR reactions were then purified using Quiagen PCR purification kit and eluted with 30 ml elution buffer.

<u>Cloning the enriched differentially expressed genes into a vector</u>

After the last amplification step of the protocol (amplification 4 see tab.4) the remaing enriched cDNA was digested with SalI and NotI as previously described and purified using Quiagen PCR amplification kit. The cDNAs were eluted with 30 ml elution buffer and 6 ml of each cDNA (up and down) was used for ligation into pSPORT 1 vector (GIBCO Brl, pre-cut with SalI and NotI). Two ml of the vector and 10U of T4 ligase (New England Biolabs) was added and incubated for 5h at room temperature.

One ml of each ligated sample (up and down) was transformed into 50 ml Chemomax efficiency DH5a competent cells (Gibco BRL) according to the manufacturers instructions.

Screening for the differentially expressed genes

After estimating the colony number per µl transformation sample, approximately 8.000 clones were plated out from each subtraction, distributed on two 22 cm^2 agar plates (4.000 colonies/plate). From these plates 6x 384 colonies were picked and placed in 384 well plates with 70 µl LB medium/well (see Maniatis *et al.*, Molecular cloning laboratory book, Appendix A.1) (+ ampicillin 100 mg/ml) using *Bio*Pick machine of *Bio*Robotics (Cambridge, UK). The bacterial clones were incubated over night at 37°C and then used for colony PCR. This PCR was performed in 384 PCR well plates in a volume of 20 µl per sample. One PCR reaction included: 2 µl 10x PCR buffer, 0,4 µl Sport-Not primer (10 pmol/µl; 5'-CGT AAG CTT GGA TCC TCT AGA GC-3' (SEQ. ID. NO. 113), 0,4 µl of Sport-Sal primer (10 pmol/µl; 5'-TGC AGG TAC CGG TCC GGA ATT CC-3' (SEQ. ID. NO. 114)), 1,6 µl dNTP mix (25 mM each), 0,4 µl 0,1% Bromphenol blue and 0,5 µl DynAzyme Taq-polymerase (2 U/µl; FynZyme). A master mix for all reactions was prepared, distributed and then inoculated with a 384 plastic replica. The PCR cycling parameters were: 2 min 94°C, 37 times (30 sec 94°C; 30 sec 50°C, 1 min 72°C) and 5 min 72°C.

Following the amplification, the PCR reactions were spotted on Hybond N+ membrane (Amersham) using Microgrid TAS of *Bio*Robotics. All clones were spotted in duplicates and genomic DNA was used as guide dots. On one filter all 6x384 up-regulated genes and all 6x384 down-regulated genes were positioned. 24 duplicates were made for analyses by hybridisation with different radioactive cDNA probes.

Hybridisation conditions: ...

Identification of the differentially expressed genes

Phospho-imager Fujifilm BAS 1800II with BAS 1800 III R program, Arrayvision version 6.0 (Imaging Research Inc), sequencing, BLAST analysis

Confirmation of the differential expression

RT-PCR...

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention as set forth in the claims appended hereto.

SEQUENCE LISTING

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DIECKMANN, Andreas
LÖFBERG, Robert
VON STEIN, Oliver
VON STEIN, Petra

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attgaa
426

Claims

1. An isolated and purified nucleic acid molecule comprising a polynucleotide selected from

(a) a nucleotide sequence at least 70 % identical to the sequence of SEQ. ID. NO. 1,

(b) a nucleotide sequence complementary to the sequence of (a),

(c) a nucleotide sequence which hybridises under stringent conditions to the sequence of (a),

(d) a nucleotide sequence functionally homologous to the sequence of SEQ. ID. NO. 1.

2. An isolated and purified nucleic acid molecule according to claim 1(a), **characterized** in that the sequence exhibits at least 80 % homology to the sequence of SEQ. ID. NO. 1.

3. An isolated and purified nucleic acid molecule according to claim 1(a), **characterized** in that the sequence exhibits at least 90 % homology to the sequences of SEQ. ID. NO. 1.

4. An isolated and purified nucleic acid molecule according to claim 1, **characterized** in that the polynucleotide is a DNA molecule.

5. An isolated and purified nucleic acid molecule according to claim 1, **characterized** in that the polynucleotide is a cDNA molecule.

6. An isolated and purified nucleic acid molecule according to claim 1, **characterized** in that the polynucleotide is a RNA molecule.

7. A PNA molecule capable of hybridising to a nucleic acid molecule according to claim 1.

8. A method of producing a recombinant vector, **characterized** in that the isolated nucleic acid of claim 1 is functionally inserted into a vector.

9. A recombinant vector produced by the method of claim 8.

10. A recombinant vector, **characterized** in that the isolated nucleic acid of claim 1 is operatively linked to regulatory sequences which control the expression of said nucleic acid sequence.

11. A recombinant vector, **characterized** in that the isolated nucleic acid of claim 1 is operatively linked to regulatory sequences which, due to the intrinsic orientation of the said nucleic acid, produces an antisense nucleic acid that's is complementary to the target sequences and hybridizes to the target sequence.

12. A method of producing a recombinant host cell, **characterized** in that the recombinant vector of claim 9 or 10 is introduced into a host cell.

13. A recombinant host cell produced by the method of claim 12.

14. A transgenic non-human animal, **characterized** in that said animal carries at least one sequence according to claim 1 operationally inserted in at lease one cell.

15. A method of screening a human subject for susceptibility to inflammatory diseases, in particular but not limited to an inflammatory bowel disease, **characterized** in said method comprising a step of screening for the presence or absence of the expression of, or the degree of expression of a sequence according to claim 1.

16. A method of diagnosing an inflammatory disease in a human subject, in particular but not limited to an inflammatory bowel disease, **characterized** in that the degree of expression of a sequence according to claim 1 is determined.

17. A compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule identified by comprising the sequence of claim 1, **characterized** in that said compound hybridises with and inhibits the expression of said nucleic acid molecule.

18. The compound according to claim 17, **characterized** in that the compound comprises 8 to 24 nucleobases in length.

19. The compound of claim 17, **characterized** in that said compound is an antisense oligonucleotide.

20. The compound of claim 17, **characterized** in that said compound has a sequence comprising at least an 8-nucleobase portion of SEQ. ID. NO. 1.

21. The compound of claim 19, **characterized** in that said antisense oligonucleotide comprises at least one modified nucleobase.

22. The compound of claim 17, **characterized** in that said antisense oligonucleotide is chemically modified by substitution in a non-bridging oxygen atom of the antisense nucleic acid backbone with a moiety selected from the group consisting of methane phosphate, methyl phosphate and phosphorothioate.

23. The compound of claim 17, **characterized** in that it is an antisense agent composed of DNA or RNA or an analogue or mimic of DNA or RNA including but not restricted to the following: methylphosphonate, N3'->P5'-phosphoramidate, morpholino, peptide nucleic acid (PNA), locked nucleic acid (LNA), arabinosyl nucleic acid (ANA), fluoro-arabinosyl nucleic acid (FANA) methoxy-ethyl nucleic acid (MOE).

24. The antisense agent of claim 23, **characterized** in that said agent is a homo or heteropolymer containing combinations of the above DNA or RNA or analogues or mimics of DNA or RNA.

25. The compound of claim 22, **characterized** in that the substitution is at one or more nucleptides independently selected from the final three nucleotides at the 3' terminus and 5' terminus of said oligonucleotide.

26. The compound of claim 22, **characterized** in that the substitution occurs at any position along the entire length of said oligonucleotide.

27. The compound of claim 22, **characterized** in that said oligonucleotide comprises at least one modified sugar moiety nucleobase.

28. The compound of claim 27, **characterized** in that the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

29. A pharmaceutical composition, **characterized** in that said composition comprises a compound according to any one of claims 16 - 28 and a pharmaceutically acceptable carrier or diluent.

30. The composition of claim 29, **characterized** in that said composition further comprises a colloidal dispersion system.

31. A method of inhibiting the expression of a sequence according to claim 1 in human cells and tissues, comprising said human cells and tissues *in vivo* or *in vitro*, **characterized** in that a compound according to any one of claims 16 – 28 is used.

Abstract

Variations in the expression of SEQ. ID. NO. 1 is shown to be associated with inflammatory diseases, and in particular inflammatory bowel disease. The invention provides oligonucleotide sequences identified to have such association, and methods of using such genes or gene products as tools in research, diagnosis or prognosis, or as targets for therapeutic intervention.
